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Reprint of "The clinical impact of deficiency in DNA non-homologous end-joining"☆

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ABSTRACT

DNA non-homologous end-joining (NHEJ) is the major DNA double strand break (DSB) repair pathway in mammalian cells. Defects in NHEJ proteins confer marked radiosensitivity in cell lines and mice models, since radiation potently induces DSBs. The process of V(D)J recombination functions during the development of the immune response, and involves the introduction and rejoining of programmed DSBs to generate an array of diverse T and B cells. NHEJ rejoins these programmed DSBs. Consequently, NHEJ deficiency confers (severe) combined immunodeficiency – (S)CID – due to a failure to carry out V(D)J recombination efficiently. NHEJ also functions in class switch recombination, another step enhancing T and B cell diversity. Prompted by these findings, a search for radiosensitivity amongst (S)CID patients revealed a radiosensitive sub-class, defined as RS-SCID. Mutations in NHEJ genes, defining human syndromes deficient in DNA ligase IV (LIG4 Syndrome), XLF-Cernunnos, Artemis or DNA-PKcs, have been identified in such patients. Mutations in XRCC4 or Ku70,80 in patients have not been identified. RS-SCID patients frequently display additional characteristics including microcephaly, dysmorphic facial features and growth delay. Here, we overview the clinical spectrum of RS-SCID patients and discuss our current understanding of the underlying biology.

1. Introduction

A DNA double strand break (DSB) is a critical lesion promoting cell death if unrepaired or genomic rearrangements if misrepaired. While ionising radiation (IR) is renowned for its avid ability to induce DSBs, around 10–20 DSBs arise endogenously per cell per day. Perhaps surprisingly, cells also create DSBs, termed programmed DSBs, during developmental processes such as immune development and meiosis. Significantly, while the goal of DSB repair in somatic cells is to maintain genomic integrity and stability, the end-point of the developmental processes involving programmed DSB formation is to create genetic diversity. Amazingly despite this basic difference, the same DSB pathway is exploited to maintain genomic stability in the face of DNA damage induced DSBs and to create diversity during immune development, although the underlying processes encompass tweaks to achieve the distinct goals. The major DSB repair pathway in mammalian cells is DNA non-homologous end-joining (NHEJ). Homologous recombination (HR) also functions to rejoin DSBs but is limited to situations where a sister chromatid, which serves as the source of the homologous sequence, is present. HR, in fact, has a more significant role in promoting recovery from replication fork stalling or collapse, a process which may not involve DSB formation at all or possibly a DSB with one end. HR also functions during meiosis, which represents another situation when a sister chromosome is present. The goal of this review is to focus on the clinical manifestation of defects in NHEJ in patients. Since NHEJ functions during immune development, we will focus on the immunodeficiency observed in NHEJ-defective patients. However, NHEJ deficiency can also confer additional developmental abnormalities, including microcephaly. We aim to couple our current understanding of the underlying biology with the clinical manifestation. We will focus on the impact in patients but call upon studies using cultured cells and mouse models, as well as biochemical and structural biology approaches to provide additional insight. Since most (but not all) of the NHEJ proteins are essential for embryonic viability, the mutational changes observed in patients are frequently

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hypomorphic (they are not fully inactivating and allow residual protein function). We use the standard convention and describe such patients as being deficient in the protein; complete loss of protein function (caused by a null mutational change) results in a patient defective for the protein under discussion.

2. The process of NHEJ

Current insight into the NHEJ mechanism will be covered elsewhere in this DNA Repair Special Issue. Other detailed reviews are also available [1–3]. Here, we provide only a brief overview encompassing points relevant for considering NHEJ-deficient patients. NHEJ is initiated by binding of the Ku heterodimer to double stranded (ds) DNA ends (Fig. 1). The rapid end-binding of Ku, which arises in part from its avid capacity to bind DNA ends coupled with its high abundancy, provides prompt protection of the DNA ends from unwanted nucleolytic degradation; Ku end-binding additionally recruits the DNA-PK catalytic subunit (DNA-PKcs) generating the DNA-PK complex. DNA-PK is a phosphatidylinositol 3-kinase (PI3K)-like kinase with a kinase domain in the C-terminus [4]. Studies in vitro have demonstrated that Ku has the capacity to translocate inwards forming multimeric complexes on the DNA molecule [5]. There is, however, no evidence that this occurs in vivo

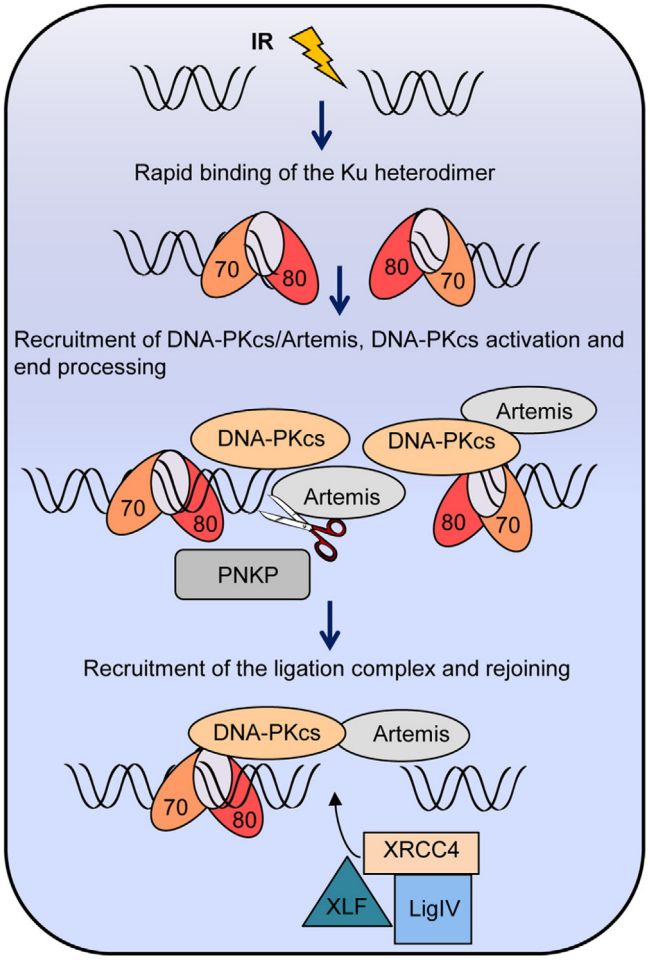

Fig. 1. Process of NHEJ. Ku (shown as a 70/80 heterodimer in yellow/red), which has avid DNA end-binding activity and is highly abundant in human cells, rapidly binds DNA ends. DNA-bound Ku recruits DNA-PKcs, which activates DNA-PK activity. DNA-PK undergoes autophosphorylation at specific clusters of sites. DNA-PK promotes steps of end-processing, which can be carried out by PNKP and, for some DSBs, by the nuclease, Artemis. DNA-PK recruits the ligation complex, encompassing DNA ligase IV, XRCC4 and XLF, which carries out the final rejoining step.

and indeed, exploitation of a recent novel approach for high resolution analysis of DNA bound proteins, has demonstrated that in vivo only a single Ku molecule is present at each DNA end (i.e. two molecules at a dsDNA end) [6]. However, DNA-PKcs recruitment may promote inward movement of the single Ku molecule, which may facilitate steps of end-processing. Assembly of DNA-PK on DNA ends activates its kinase activity [7]. Structural studies have shown that Ku is a basket shaped structure with a central core of appropriate dimensions to allow its looping onto ds DNA without necessitating interaction with nucleotide bases [8]. Ku-DNA binding is, therefore, DNA sequence independent. The structure of DNA-PKcs has revealed a flexible ring shaped molecule arising from multiple alpha-helical HEAT repeats (helix-turn-helix motifs), which facilitate bending [9]. The flexible structure and an opening in the ring endows DNA-PKcs with the capacity to fold onto the DNA molecule [10]. It is at this stage of the process that DNA-PK facilitates end-processing steps, which may involve deletion formation or "fill in" regions of ssDNA regions [11]. Ligation requires 3′OH and 5′P ends, which rarely arise following DNA damage, but are generated by a process involving polynucleotide kinase/phosphatase (PNKP). DNA-PK may promote PNKP processing by phosphorylation [12]. End-processing may also require nuclease activities. DNA-PKcs interacts with the structure specific nuclease, Artemis [13]. Artemis has a Metallo-β-lactamase domain at its extreme N-terminus and a closely localised β-Casp domain [14]. The C-terminus appears to be regulatory. Artemis, in contrast to other NHEJ proteins, appears to be uniquely required for the repair of a 15% subset of DSBs, representing those repaired with slow kinetics [15]. Moreover this process has an essential requirement for DNA-PKcs [15]. Artemis also functions to open the hairpin ended intermediates that form during the process of V(D)J recombination in a DNA-PKcs dependent manner (see further details below) [13]. In G2 phase, where HR can also function, Artemis is, as in G1 phase, required for the slow component of DSB repair – however, in G2 this slow repair process represents HR [16]. The available evidence suggests that in G2 phase, there is a defined switch from rejoining by NHEJ to the use of HR in those situations where rapid repair by NHEJ fails to ensue [17]. Thus, we propose a model in which NHEJ initially attempts to repair all DSBs by a "core" process that does not require Artemis processing but if this process stalls, then a switch to promoting resection and repair by HR occurs [17]. In contrast, Artemis is dispensable for HR following replication fork stalling/collapse. However, although Artemis is required for resection, it does not appear to be the nuclease that directly effects resection since it has the wrong polarity. We propose that in G1 phase, a similar hierarchy exists. However, in G1 phase, the slow rejoining process may represent a form of resection-mediated end-joining, such as microhomology mediated end-joining (MMEJ) rather than HR, with resection being less extensive (Lobrich and Jeggo laboratories, unpublished findings). The role of Artemis during V(D)J recombination, where a hairpin end is the barrier to core NHEJ-dependent rejoining, may reflect a similar hierarchy with a function for Artemis in opening the hairpin end [13]. It has been proposed that, at least during V(D)J recombination, DNA-PKcs facilitates Artemis activity by remodelling the DNA end [18]. Whether DNA-PKcs promotes Artemis function at damage-induced DSBs remains to be determined. These in vivo roles of Artemis are consistent with biochemical analysis showing that in the presence of DNA-PK Artemis can cleave hairpin ends and 5′ and 3′ overhangs [13,14].

Finally, DNA-PK plays an important role in recruiting the NHEJ ligation complex, which encompasses XRCC4, DNA ligase IV and XLF [19]. Recent studies also suggest that the ligation complex may promote DNA-PKcs autophosphorylation, and potentially the release of DNA-PK from the end [20]. Nonetheless, it is currently unclear when removal of the DNA-PK complex occurs and whether

it precedes or follows ligation. DNA-PK autophosphorylation has been proposed as a mechanism promoting the assembly and disassembly of the DNA-PK complex on the DNA end [21]. However, at least from in vitro studies, Ku remains attached to the DNA end [10]. If ligation occurs prior to loss of Ku, then there must be degradation of Ku since its structure does not allow its release from an intact DNA molecule [8]. RNF-8-dependent ubiquitylation has been proposed as a potential mechanism regulating the removal of Ku following DSB rejoining [22].

DNA ligase IV has a conserved ligase domain located at its N-terminus, which can be subdivided into a DNA-binding and an adenylation domain [23,24]. A tandem BRCA1 C-terminus (BRCT) domain is located in the C-terminus of DNA ligase IV. XRCC4 is a homodimer and binds a single DNA ligase IV molecule [25]. XRCC4 has a globular head domain and helical tails that form a coiled coil that unwinds upon ligase binding. The region between the two BRCT motifs of DNA ligase IV and part of the second BRCT motif are required for binding to XRCC4 [25,26]. DNA ligase IV and XRCC4 interact tightly and regulate one another's stability [27–29]. XLF, in contrast, is a weaker binding component of the ligation complex [30]. XLF is structurally related to XRCC4 but crucially has a shorter coiled coil domain and undergoes a reverse in direction, precluding the critical "kink" in the coiled coil that is required for interaction with DNA ligase IV [31,32]. Several recent studies have proposed that XRCC4 and XLF form a protein filament along the DNA, that serves to bridge the DNA ends, suggesting a role in synapsis [33–35]. Further, studies, however, are required to verify that such structures form in vivo.

3. The exploitation of NHEJ during the development of the immune response

While the evolutionary pressure on NHEJ in the repair of damage induced DSBs is to maintain genomic stability and avoid sequence changes, the development of the immune response is driven by the need to generate genetic diversity to produce a broad repertoire of different immunoglobulin and T cell receptor genes that are able to recognise the wide array of distinct antigens to which we are exposed. In germ line cells, the components of the immunoglobulin and T cell receptor genes are sequentially arranged as distinct arrays of variable (V), diversity (D) or joining (J) segments [36]. During immune development, the gene segments undergo rearrangement via the process of V(D)J recombination to produce distinct re-arrangements of V, D and J sub-fragments. The process of V(D)J recombination has, like NHEJ, been described in several excellent reviews and only an outline will be given here [36–38]. In germ line cells, each V, D or J segment has a coding region, C, and a flanking recombination signal sequence (RSS). V(D)J recombination is initiated by cleavage at each of two RSS sequences by the RAG1/2 recombinases, thereby generating a synapsis complex that encompasses two hairpin-ended and two blunt ended DSBs. The blunt ends form at the RSSs and rejoin by a process that requires the core NHEJ proteins (Ku, DNA-PKcs, XRCC4, XLF and DNA ligase IV but not Artemis) to yield either circles or inversions of the RSS sequences (Fig. 2). The ends of the coding sequences, which encode segments of the immunoglobulin and T cell receptor genes, arise as hairpins and require cleavage by Artemis prior to rejoining by core NHEJ proteins. Following Artemis cleavage, which does not occur at the apex of the hairpin, nucleotide additions, fill-in or deletions can arise by processes involving terminal nucleotide transferase (TdT), and polμ or λ polymerases. These end modifications add further to the diversity of the immunoglobulin and T cell receptor genes.

In addition to V(D)J recombination, DSB formation also forms during immunoglobulin class switch recombination (CSR), an additional process that allows switching of immunoglobulin isotype subclasses. Since CSR necessitates that V(D)J recombination has taken place, the more marked phenotype of mouse models and human patients deficient in NHEJ proteins is observed as diminished V(D)J recombination. However, studies in animal models in which a rearranged substrate is engineered into cells has revealed the role for NHEJ proteins in CSR. Moreover, in mild NHEJ-deficient human phenotypes, a defect in CSR can be observed and can present as markedly raised IgM, but diminished or absent IgA and IgG. Here, however, we will focus on V(D)J recombination given that reduced or absent T and B cells is the more common phenotype observed in NHEJ deficient patients.

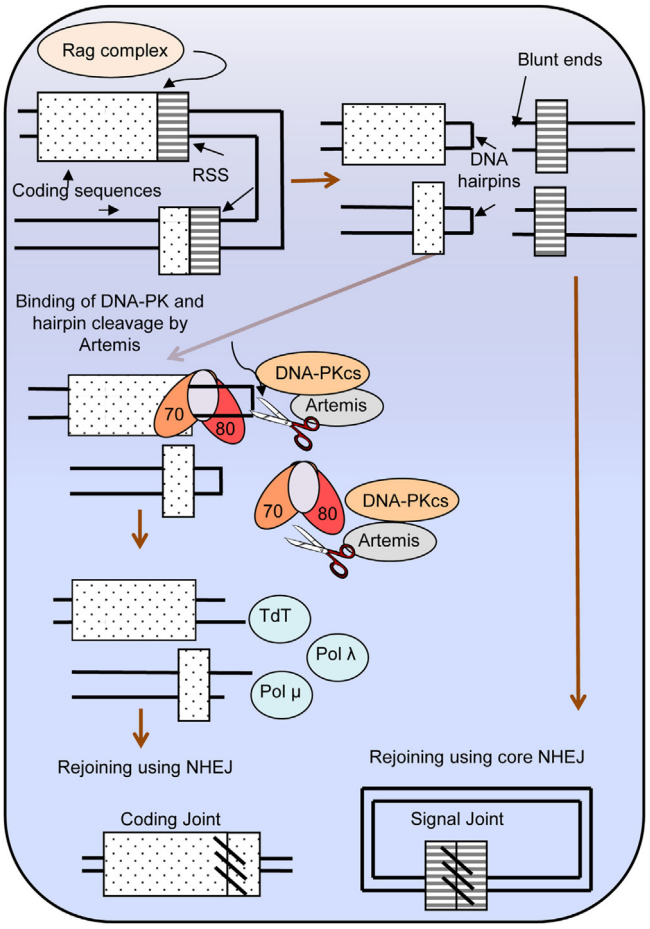

Fig. 2. Utilisation of NHEJ during V(D)J recombination. V, D and J segments are sequentially organised in genomic DNA and undergo rearrangement in T and B cells during V(D)J recombination. The process is initiated by site specific DSBs introduced by the RAG1/2 recombinases directed by the RSS sequences. The coding ends form as hairpins whilst the (signal) RSS ends are blunt ended. The DNA-PK complex assembles at both the hairpin and blunt ends in a similar manner to the process shown in Fig. 1. The signal ends are rejoined by simple NHEJ without requirement for Artemis. In contrast, Artemis is required to cleave the hairpins arising at the coding ends. Artemis cleaves non-apically and subsequent end processing involving nucleotide insertions by TdT, as well as potentially by Polλ and polμ helps to generate ends with deletions and/or insertions.

4. The radiosensitive, severe combined immunodeficiency (RS-SCID) phenotype

As a consequence of the role of NHEJ in the rejoining of radiation-induced DSBs and the programmed DSBs induced during V(D)J recombination, patients deficient in NHEJ proteins display radiosensitivity and severe combined immunodeficiency (SCID), a phenotype which has been called RS-SCID. To date, mutations

Table 1
Clinical presentation and features of LIG4 Syndrome patients. All mutations are compound heterozygous unless specified as homozygous ([H]).

Patient	Immune phenotype	Developmental delay/microcephaly	Cancers	Mutation	Reference
180BR	No over immunodeficiency	X/X	Acute lymphoid leukaemia	p.R278>H[H]	[43]
CoDA	Mild immune deficiency	X/X	Squamous cell carcinoma	p.R580X/p.R814X	[104]
495BR	CID, neutropenia	X/X	X	p.R278>P/p.E427>A	[30]
P2	Neutropenia	√/X	X	p.H282>L/p.D423X	[105]
F07/614	CID	X/√	X	p.A3>V/p.T9>I/p.R278>H	Unpublished
P1	Neutropenia	X/√	X	p.H282>L/p.D423X	[105]
P1/P2	B and T cell lymphocytopenia	X/√	X	p.Q280>R/p.K424FS	[47]
411BR,	Pancytopenia	√/√	B cell lymphoma	p.A3>V/p.T9>I/p.R278>H[H]	[39,100,50]
NBS2303,	Pancytopenia	√/√	X	p.R814X/p.R580X,	
NBS2304,	Pancytopenia	√/√	X	p.R814X/p.R580X,	
99P0149	Pancytopenia	√/√	X	p.R814X/p.G469>E	
SC2	T-B-NK+ SCID	X/X	X	p.Q433Δ[H]	[46]
P1	Progressive CID	X/X	EBV-non-Hodgkins lymphoma	p.M249V/nucleotide position 1270–1274Δ	[49]
P1	SCID with features of Omenn's Syndrome	X/X	X	p.H282L[H]	[48]
P1	Pancytopenia, hypo gammaglobulinaemia	√/√	X	p.A797DfsX2/p.R814X	Unpublished

in four genes encoding components of the NHEJ machinery, *LIG4* (encoding DNA ligase IV), *XLF* (encoding XLF), *PRKDC* (encoding DNA-PKcs) and *DCLRE1C/SNM3/Artemis* encoding Artemis) have been identified in patients (Figs. 3–6). Although RS-SCID represents the extreme phenotype, radiosensitivity coupled with variable immunodeficiency ranging from Omenn's Syndrome to CID or pancytopaenia, or progressive immunodeficiency has been observed. Additional features are also observed in some patients, including microcephaly and severe growth/developmental delay (Figs. 3–6). The clinical manifestation arising from deficiency in each NHEJ component will be described below and, where possible, considered in the light of our knowledge of the protein's structure and role in NHEJ.

4.1. LIG4 Syndrome

LIG4 Syndrome (OMIM606593), caused by mutations in DNA ligase IV, was the first genetically defined RS-CID disorder to be described [39]. Disruption of the *DNA ligase IV* (*Lig4*) gene (resulting in complete loss of DNA ligase IV function) causes embryonic lethality in mice with extensive neuronal apoptosis around E13.5 [40–42]. Thus, DNA ligase IV is essential for embryonic viability. Consequently, all mutations identified in patients are hypomorphic (i.e. not fully inactivating). The range of features observed in patients and their mutational changes are given in Table 1; Fig. 3 overviews the sites of the mutational changes observed. The first patient mutated in *Lig4* to be identified had no overt phenotype (i.e. no obvious immunodeficiency) but developed acute lymphoblastic leukaemia at age 14. The patient subsequently dramatically over-responded to cranial radiation treatment and died from radiation morbidity [43,44]. This patient proved to have a homozygous R278H missense mutation which reduced DNA ligase IV activity to ~10% of the control level. Significantly, the mutation is located close to the ATP binding site and is predicted to impede formation of a DNA ligase IV-adenylate complex [45]. All subsequent patients have displayed more marked immunodeficiency although in most cases low but residual T and B cells are present (i.e. a CID phenotype) (Table 1). However, some patients with a more pronounced SCID phenotype have been described and one patient displayed features of Omenn's Syndrome [46–48]. Microcephaly (a reduced head circumference), growth delay and dysmorphic facial features are also commonly observed in patients (Table 1) [39]. Significantly, microcephaly is observed at birth but is not progressive post-natally. Some additional sporadic developmental anomalies have also been described in some patients, such as bone abnormalities [39]. Lymphoid tumours have been observed in a small number of patients, usually those with milder immunodeficiency, including the mild patient described above who displayed no overt immunodeficiency but developed leukaemia (Table 1) [44,49,50]. In all cases examined, patient fibroblasts show radiosensitivity and diminished DSB repair capacity, which can be observed from early times post exposure (Fig. 7). Since the mutations are hypomorphic, however, residual DSB rejoining occurs with slow kinetics and normally by 24–72 h post exposure all DSBs have been rejoined. Interestingly, from our studies we have observed that the magnitude of DSB capacity correlates with the severity of clinical features. Thus, fibroblasts derived from the patient with the mildest features (i.e. no overt immunodeficiency or microcephaly) showed a greater DSB repair capacity compared to fibroblasts derived from patients with more severe features [51]. Interestingly, in this context, an analysis of two cell lines with homozygous changes at R278H, revealed that two additional linked polymorphic changes in one patient correlated with a more severe phenotype both clinically and at the cellular level [51].

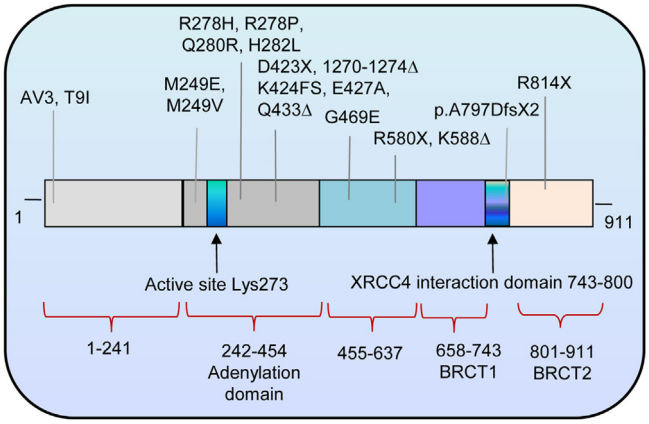

Fig. 3. Location of mutational changes identified in LIGIV Syndrome patients. *DNA ligase IV* cDNA has a ligase domain in its 5′ region, which includes a DNA binding domain (DBD) and an adenylation domain. The 3′ region encompasses a tandem BRCT domain with a spacer region. The position of the mutations identified in patients is shown. AV3 and T9I represent two closely linked polymorphic changes, which have been reported to impact upon DNA ligase IV activity but have been found together with the R278H change in patients.

Table 2
Clinical presentation and features of XLF-deficient patients. All mutations are homozygous unless specified as compound heterozygous ([CH]). GR, growth delay; MCPH, microcephaly; CID, combined immunodeficiency; SCID, severe combined immunodeficiency.

Patient	Immune phenotype	Developmental phenotype	Mutation	Reference
2BN	SCID	Small statue MCPH	p.R178X	[54]
P1	CID	GR/MCPH	p.R57G/p.C123R[CH]	[47]
P2	CID	GR/MCPH	p.R178X	[47]
P3	CID	GR/MCPH	p.G267Δ	[47]
P4	CID	GR/MCPH	p.G267Δ	[47]
P5	CID	GR/MCPH	p.R57G	[47]
P1	?	Polymicrogyria	Chromosomal translocation t(2;7)(q35;p22)	[106]
F07/402	SCID	Developmental Delay/MCPH	p.R57X	[30]
P1	Immune deficiency	MCPH	p.R57G	[107]
P2	Mild immune deficiency	MCPH	c.495dupA/Δ1.9kb[CH]	[107]
P3	?	MCPH	c.495dupA/Δ1.9kb[CH]	[107]
P4	Autoimmunity	MCPH	p.R176X	[107]
P5	?		p.R178X	[107]
P1	CID, macrocytic anaemia, neutropenia	GR/MCPH	p.R57G	[108]
P1	SCID	GR MCPH	p.R178X	[109]

The mutational changes identified in LIG4 Syndrome patients are located throughout the gene and most changes are predicted to impact upon function (Fig. 3). The point mutational changes observed are frequently localised within the adenylation domain, with structural analysis predicting an impact on adenylate complex formation [45]. Interestingly, although the severity of microcephaly normally correlates with the degree of immunodeficiency, one patient with a pronounced SCID phenotype displayed no microcephaly or growth delay providing evidence that these phenotypes can be separated [46]. Further discussion of the basis underlying microcephaly in these patients is given in section 5 below.

4.2. XLF/Cernunnos defective patients

RS-SCID was described as a phenotype in a number of immunodeficiency patients but identifying the defective gene(s) progressed slowly [52–54]. The identification of mutations in *DCLRE1C/Artemis* was the next genetic defect for RS-SCID to be identified, facilitated by its occurrence within a community of Athabascan America Indians (see below for further details) [55]. Two distinct subsequent approaches led to the identification of XLF (also called Cernunnos or NHEJ1) as a further defect causal for RS-(S)CID (OMIM611291). In one approach, a panel of cell lines from RS-(S)CID patients displaying growth retardation, microcephaly, immunodeficiency and cellular radiosensitivity was exploited to identify *XLF/Cernunnos* as a cDNA able to complement the radiosensitivity phenotype [56]. A second approach centred on identifying proteins interacting with DNA ligase IV/XRCC4 revealed the same factor [31]. Significantly, XLF proved to be the homologue of Nej1, another NHEJ factor that was identified from studies in yeast [57–59]. Thus, XLF is additionally called NHEJ1.

Microcephaly and growth delay is a feature observed in all XLF patients (Table 2). In contrast to the situation with DNA ligase IV and XRCC4, disruption of *XLF* in mice does not appear to confer embryonic lethality, although XLF-defective mice display spontaneous genomic instability and elevated translocations [60]. Although it is difficult to substantiate whether the mutational changes in patients confer loss of function mutations, several *XLF* mutational changes do appear to fall into that category, with some representing severe truncating mutations [30]. The available data suggest that XLF may not be essential for NHEJ although its loss profoundly impacts upon the process [30]. Despite the fact that XLF appears to be non-essential for NHEJ whilst DNA ligase IV is essential, clinically XLF patients resemble the more severe LIG4 Syndrome patients (compare Tables 1 and 2). Thus, inactivation of XLF confers a clinical manifestation that is similar to partial loss of DNA ligase IV. XLF-defective patient fibroblasts also display a similar level of radiosensitivity and magnitude of impaired DSB repair to that observed in LIG4 Syndrome patients (Fig. 7). The mutational changes identified in XLF patients are shown in Fig. 4.

4.3. Artemis-defective patients

Loss of Artemis represents the most common cause of RS-SCID (OMIM602450) [55]. This is likely because although Artemis has an essential role in opening the hairpin intermediate during V(D)J recombination, it does not appear to have any other essential developmental role and indeed knock-out mice are viable (see below for further discussion) [61]. As discussed above, the role of Artemis in NHEJ is distinct to other "core" NHEJ components, since Artemis is essential for the rejoining of a ~15% subset of radiation induced DSBs [15]. Consequently, exposure of null-Artemis patient cells to X or γ-Rays results in a fraction of DSBs remaining unrepaired for prolonged periods (up to 21 days), which is distinct to the defect observed in LIG4 Syndrome or XLF-defective patient cells, where all DSBs are repair with slow kinetics (Fig. 7). Thus, by 1–7 days post irradiation (3 Gy) all DSBs are rejoined in LIG4 Syndrome and XLF-defective patient cells. This specific requirement of Artemis for a subset of DSB rejoining is not a unique feature of radiation-induced DSBs, however, since a similar phenotype is observed following exposure to oxidative damage or prolonged maintenance of Artemis cells without subculturing where oxidative damage can

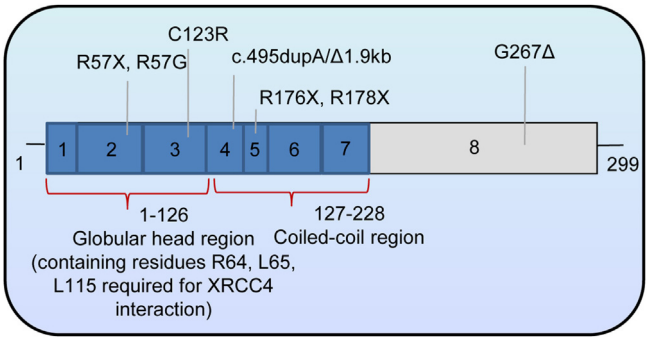

Fig. 4. Location of mutational changes identified in XLF-Cernunnos deficient patients.

accumulate [62]. Similarly, Artemis is essential for cleaving the hairpin intermediate generated during V(D)J recombination but is dispensable for the rejoining of the blunt-ended RSS ends [13]. Further discussion in the context of neuronal development is provided below.

4.3.1. Clinical manifestation of hypomorphic mutations in Artemis

A substantial subset of Artemis patients have genomic deletions that result in loss of the transcriptional start site of *Artemis* cDNA; cells from such patients display no detectable *Artemis* cDNA and no detectable expression of Artemis protein [63]. Such Artemis null patients present with SCID, with an absence of T and B cells, necessitating bone marrow transplantation. It is notable that this SCID phenotype is more marked than most LIG4 or XLF-deficient patients, who show reduced but residual T and B cells (CID or pancyotpaenia). Microcephaly, growth delay or dysmorphic facial features have not been observed in Artemis null patients, although growth can be slow due to persistent infections and the consequent health impact [55]. The classical Artemis null phenotype will not be discussed in further detail here since this has been covered in excellent reviews [64,65].

Here, we will focus on reviewing a class of Artemis-deficient patients, termed "leaky" Artemis deficiency, whose importance is becoming increasingly recognised (Table 3) [66,67]. Perhaps unsurprisingly, given that the level of residual Artemis expression may cover a range, the phenotype of such patients can be broad (Table 3). Interestingly, such patients frequently display progressive immunodeficiency. One patient displayed Omenn's Syndrome, due to oligoclonal expansion of a reduced number of T or B cells [68]. Interestingly, lymphomas, often EBV-associated, can be observed in such patients, which is rarely seen in Artemis-null patients due to their lack of B cells [55,69]. A similar phenotype is also observed in mouse models for Artemis deficiency [70,71]. This suggests that a small number of aberrant recombination events allow the expression of oncogenes either directly or via an EBV-associated process. One interesting, late onset Artemis-deficient patient with a homozygous polymorphism in Artemis observed as a heterozygous change in ~1% of the Asian community displayed mild immunodeficiency [72]. The polymorphism was impacting on Artemis activity. Significantly, the patient developed progressive immunodeficiency and non-lymphoid carcinomas. Since this was a single patient, however, it is difficult to assess the causal significance. An important area of future work is to establish the contribution of Artemis-deficiency to mild or progressive immunodeficiency, as well as to lymphoid as well as non-lymphoid cancers. The distribution of mutations found in hypomorphic Artemis patients in shown in Fig. 5.

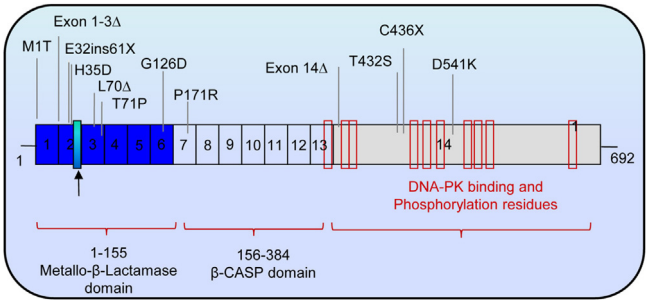

Fig. 5. Location of Mutations identified in Artemis in hypomorphic patients. *Artemis* cDNA has a metallo-β lactamase and a β-CASP domain in its 5′ region and a regulatory region, with phosphorylation sites at its 3′ end. The sites of mutations identified in hypomorphic mutations are shown. Note that some of these changes are likely to be inactivating changes (e.g. exon 1–3Δ) since the hypomorphic change may only be in one allele. We have not distinguished the hypomorphic alleles since they has not be identified in all cases.

4.4. Defects in DNA-PKcs

Loss of DNA-PKcs in mice confers SCID but no additional phenotype, similar to that observed following loss of Artemis [73,74]. Thus, for many years it was an enigma that SCID or CID patients with mutations in *DNA-PKcs* had not been identified. In 2009 a DNA-PKcs deficient patient was reported with features closely resembling those of Artemis null patients (i.e. SCID without any other developmental impact) [75]. Interestingly, the mutational changes did not impact upon DNA-PK kinase activity but rather on Artemis function. The fact that the mutational change had a subtle impact upon DNA-PK strengthened the emerging notion that DNA-PKcs may have a more significant developmental function in humans compared to mice [76]. This notion emerged from studies involving the disruption of Ku or DNA-PKcs in human cell lines, representing a substantial and important undertaking by the Henderson laboratory. Significantly, these studies provided strong evidence that Ku and DNA-PKcs have roles in telomere maintenance in human cells, which is not reflected in animal models; Ku is essential for viability in human but not rodent cells and loss of DNA-PKcs, although not essential, has a marked impact on proliferative capacity [77,78]. The available evidence suggested that the essential function may lie in telomere maintenance. More recently, another DNA-PKcs deficient patient with a dramatic neuronal phenotype was described [79]. In this case, two heterozygous mutational changes resulted in a marked impact on DNA-PK protein level and activity, with only 1% of the normal DNA-PKcs being detectable and a very low level of activity being observed in an assay for V(D)J recombination (Fig. 6). The patient displayed marked microcephaly, developmental delay and, in contrast to LigIV Syndrome and XLF-deficient patients, neuronal degeneration was progressive post natally. There was no evidence that the mutational changes conferred a dominant negative phenotype, strongly suggesting that DNA-PK has a major role during neuronal development (pre and post birth) in humans but not in mice. Whilst it is possible that the underlying cause of this phenotype is an inability to maintain telomere length, the marked neuronal impact is not entirely consistent with such an explanation. This will be discussed in more detail in section 6.

5. Related radiosensitive, immunodeficiency disorders

The presence of DSBs also initiates a signalling response, which causes extensive changes to the chromatin in the DSB

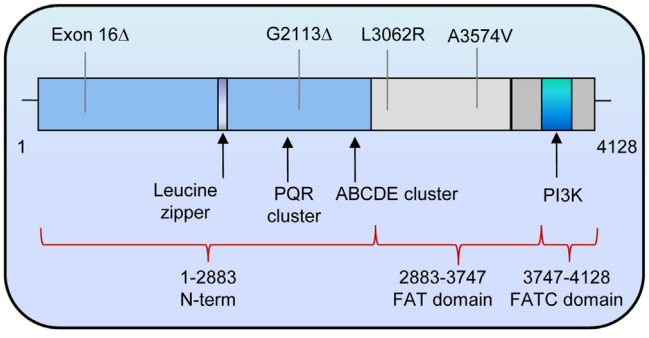

Fig. 6. Mutations identified in DNA-PKcs in patients. Two isoforms of DNA-PKcs have been described. The larger isoform (Isoform 1, Accession: NP_008835.5) is 4128 amino acids in length; the less common, smaller isoform (isoform 2) has a small deletion in the C-terminal region (Accession: NP_001075109.1) and is 4097 amino acids in length. *DNA-PKcs* cDNA has a P13K-like kinase domain in its extreme 3′ region and additional HEAT and FAT domains (only the FAT domains are shown). There are two clusters of phosphorylation sites. Only two DNA-PKcs deficient patients have been identified and the mutational changes are shown.

Table 3
Clinical presentation and features of hypomorphic Artemis-deficient patients. All mutations are compound heterozygous unless specified as homozygous(H).

Age at onset	Failure to thrive	Recurrent infections	Neutropenia	Hyper IgM syndrome	Autoimmunity	Aneamia	Lymphopenia	Omenn's Syndrome	EBV-associated lymphoma	Carcinoma	Mutation	Artemis expression and V(D)J recombination	Outcome	Reference
Birth	X	√	X	X	√	√	√	X	√	X	Exon 1-3Δ/p.D451K	Unknown	Died of EBV lymphoma	[69]
Birth	X	√	X	X	X	X	√	X	√	X	Exon 1-Δ3/p.D451K	Low-level V(D)J	Died of EBV lymphoma	[69]
Birth	X	√	X	X	√	√	√	X	X	X	Exon 1Δ-3/p.T71P	Unknown	Successful transplantation	[67]
5 mth	√	X	X	X	X	X	X	√	X	X	p.M1T/p.H35D	Reduced expression, activity and V(D)J	Successful transplantation	[68]
6 mth	X	√	X	X	√	√	√	X	X	X	p.L70Δ/p.G126D	Reduced expression, normal V(D)J	Died of HSCT complications	[110,111]
9 mth	√	√	X	X	X	X	√	X	X	X	c.464+1g>a/c.464+1g>a(H)	Residual V(D)J	Successful transplantation	[112]
10 mth	X	√	√	X	√	√	√	X	X	X	Exon 1Δ-3/p.T71P	Unknown	Died of aspergillosis and BMT complications	[67]
1 yr	X	√	X	X	X	X	√	X	X	X	Exon 1-3Δ/p.D451K	Low-level V(D)J	Died of sepsis and respiratory failure at 13	[69]
2 yr	X	X	√	X	X	X	√	X	√	X	p.P171R/p.C436X(H)	Reduced	Successful transplantation	[72]
4 yr	X	√	X	X	X	X	√	X	X	X	p.E32ins61X/p.E32ins61X	Reduced expression, defective V(D)J	Successful transplantation	[113]
4 yr	√	√	X	X	X	X	√	X	X	X	p.T432S(H)	Reduced V(D)J	Died of liver cirrhosis at 16	[69]
5 yr	X	√	X	X	X	X	√	X	X	X	c.464+1g>a/c.464+1g>a	Reduced expression, residual activity, defective V(D)J	Successful transplantation	[113]
5 yr	X	√	X	√	X	X	X	X	X	X	Exon 14Δ	Unknown	Died of septic shock at 6	[114]
27 yr	X	√	√	X	X	X	√	X	X	√	p.P171R/intronic insertion= abnormal splicing	Reduced expression	On-going case	[72]
?	X	X	X	X	√	X	X	X	X	X	p.M1T/p.H35D	Unknown	Died of aspergillosis	[68]

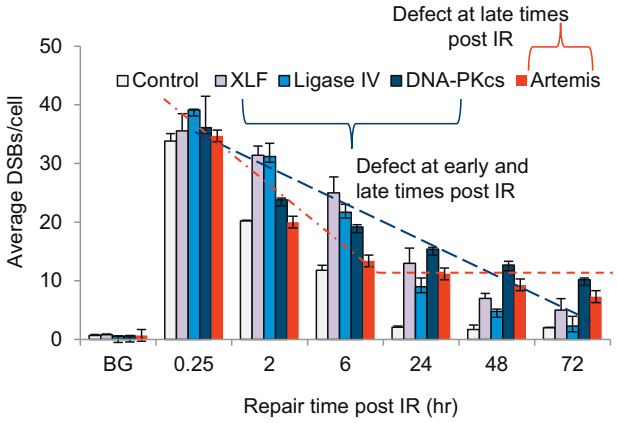

Fig. 7. Distinct in DSB repair profiles by patient cells mutated in different NHEJ proteins. γH2AX provides a sensitive procedure to monitor radiation induced DSB formation and repair in G0 (serum starved) human fibroblasts derived from patients with mutations in the indicated genes. The results represent the analysis of a single patient cell line but are reflective of the changes observed in multiple patients (except for DNA-PKcs, where only a single patient line is analysed). In control fibroblast, DSB repair occurs with fast and slow kinetics. Cells deficient in DNA ligase IV or XRCC4 rejoin all their DSBs with slow kinetics (NB that the *x*-axis is not linear so the line drawn as linear loss of DSBs does not necessary reflect a linear process). Cells defective in Artemis, repair DSBs with the same kinetics as control cells up to ~6 h post irradiation, but fail to rejoin those DSBs repaired with slow kinetics in control cells. The cell line deficient in DNA-PKcs has a partial defective in the fast component of DSB repair as well as a marked defect in the slow component. BG represents the background level of DSBs in the cells prior to radiation.

vicinity and activates processes, such as cell cycle checkpoint arrest, which influence but are not essential for NHEJ. The kinase, ataxia telangiectasia mutated (ATM), is central to the DSB signalling response. Additionally, the MRE11/RAD50/NBS1 (MRN) complex is required for the ATM signalling response, at least in part, due to a role in activating and recruiting ATM. Human disorders defective or deficient in ATM (ataxia telangiectasia; A-T; OMIM208900), MRE11 (AT-like disorder; ATLD; OMIM604391), RAD50 (RAD50 deficiency; OMIM613078) and NBS1 (Nijmegen Breakage Syndrome; NBS; OMIM251260) have been described and patients belonging to these disorders display radiosensitivity and variable levels of immunodeficiency [80–83]. Although, a detailed description of these disorders falls outside the scope of this review, they are briefly discussed here for comparison. The signalling response to DSBs has only a modest impact on the overall level of DSB repair with ATM and the MRN component proteins only being essential for a subfraction (~15%) of NHEJ, representing those DSBs repaired with slow kinetics [15]. However, multiple studies have demonstrated a more qualitative effect of these proteins on the fidelity and/or efficiency of DSB rejoining [84]. A broader impact on the rejoining process is particularly evident for the processes of V(D)J recombination and CSR, where, although SCID is not a common feature of the disorders, a compromised immune response is clearly evident in patients and multiple studies have demonstrated that loss of ATM or MRN proteins influences the nature, including the accuracy, of the V(D)J junctions [37,85,86].

A further distinct but overlapping impact lies in the developmental features of the patients. Importantly, A-T and ATLD patients display progressive ataxia and neurodegeneration but do not show microcephaly at birth like many of the NHEJ patients [84]. In contrast, NBS patients display microcephaly and dysmorphic facial features like the NHEJ patients but not progressive ataxia [82]. This distinction has been further confused by a recent description of MRE11-deficient patients with features of NBS (i.e. microcephaly) and not progressive ataxia [87].

6. How does NHEJ deficiency confer microcephaly and growth delay

The immunodeficiency conferred by defects in NHEJ is readily explained by the well described role of NHEJ in rejoining the programmed DSBs introduced during both V(D)J recombination and class switch recombination. The basis underlying the additional developmental impacts of NHEJ deficiency is less clear, however. The most frequent phenotypes are microcephaly and dysmorphic facial features, with less commonly growth or developmental delay being observed, and occasional additional features such as bone abnormalities. Since most insight has been gained into the role of NHEJ during neuronal embryogenesis, we will focus on that function. With the exception of the severe DNA-PKcs patient described above, the microcephaly observed in most patients is evident at birth but is not progressive. The study of mice knocked out for DNA ligase IV and two hypomorphic mouse models for LIG4 Syndrome, one of which involved the knock-in of a human mutational change (R278H) observed in patients, have provided most insight into the role of NHEJ during neurogenesis [40–42,88–90].

The embryonic neuronal stem and progenitor cells are located in the ventricular and sub-ventricular zones (VZ-SVZ) which lie adjacent to the ventricular membrane [91,92]. Adjacent to the VZ-SVZ is the intermediate zone (IZ) with the cortical plate (CP) representing a distal layer observed during later stages of neurogenesis. The VZ-SVZ cells undergo extremely rapid cell division from mouse developmental stage E8 to E16.5, after which cell division ceases. Although there are stem/progenitor cells in the adult brain, their rate of cell division is much reduced compared to this embryonic stage. At early stages, symmetrical cell division occurs with each stem cell giving rise to two daughter stem cells, a process that is crucial to the accumulation of a sufficient pool of stem cells to provide the large number of neurons required for normal neuronal development [93]. At a later stage, there is a switch to asymmetric division, with each stem cell generating one daughter stem cell and one progenitor cell, which will differentiate to become a neuron [94]. The VZ-SVZ compartment can be readily distinguished from the IZ region and the CP by position relative to the ventricle and the ventricular membrane. Importantly, the VZ/SVZ cells are exquisitely sensitive to apoptosis; indeed apoptosis can be activated in the VZ/SVZ by <0.5 Gy radiation whilst apoptosis is not induced even by high doses (>10 Gy) in the adult brain [95]. Apoptosis is also sensitively activated in the IZ compartment, although less readily than in the VZ/SVZ [96]. Importantly, DNA ligase IV null embryos die between E13.5 and E16.5 from massive neuronal apoptosis, suggesting that endogenous DSBs may arise from the rapid cell division at this developmental stage [40,97]. Loss of p53 relieved both the neuronal apoptosis and embryonic cell death conferred by loss of DNA ligase IV [97]. Studies on neurogenesis using a mouse model for LigIV Syndrome (*LigIV*^{Y288C}) showed small heads (as well as small bodies) and elevated apoptosis in the VZ/SVZ and IZ compartments [95]. Although exposure of wild type embryos to radiation resulted in a greater level of apoptosis in the VZ/SVZ compared to the IZ, the level of endogenous apoptosis in the *LigIV*^{Y288C} embryos was greater in the IZ compartment. Increased levels of unrepaired DSBs correlated with the increased endogenous apoptosis. Collectively, the analysis suggested that two factors contribute to the marked impact of loss of DNA ligase IV during neurogenesis. Firstly, the rapid replication from E8 to E16.5 causes enhanced DSB formation, which require NHEJ for repair. Secondly, there is a low threshold for activating apoptosis in the VZ/SVZ and IZ such that apoptosis is activated by a small elevation in numbers of unrepaired DSBs (<5). Whilst unrepaired DSBs most likely arise in other tissues due to diminished NHEJ capacity, the impact appears greater in the embryonic neuronal stem/progenitor cell compartment due to the exquisite sensitivity

for activating apoptosis. As mentioned above, a surprising finding was the enhanced level of apoptosis in the IZ compared to the VZ/SVZ compartment in *LigIV*^{Y288C} embryos. A potential explanation is that diminished NHEJ capacity allows the persistence of DSBs; thus, cells harbouring DSBs may progress from the VZ/SVZ into the IZ; in contrast, the induced DSBs in an irradiated wild type mouse are likely to undergo rapid repair. Interestingly, the G2/M damage-induced checkpoint in the embryonic brain is not activated by low numbers of DSBs, potentially allowing cells with DSBs that arise in the VZ/SVZ to progress into the IZ compartment [95].

As mentioned above, Artemis-null patients do not display microcephaly and Artemis-null mice are developmentally normal (apart from a SCID phenotype). As discussed above, Artemis-defective cells have a distinct DSB repair defective phenotype compared to that observed in LIG4 Syndrome cells or cells deficient in XLF: the Artemis defect results in a complete failure to repair a 15% subset of DSBs; deficiency in DNA ligase IV or XLF results in slow repair of all DSBs (Fig. 7). It is possible that the number of unrepaired DSBs in the absence of Artemis in the VZ/SVZ compartment lies below the threshold required to activate apoptosis. Another possibility is that the DSBs that arise from rapid replication are distinct to radiation induced DSBs and do not require Artemis for their repair. The current models propose that HR plays the major role in repairing replication-associated DSBs; thus, a role for NHEJ is perhaps surprising. It is, therefore, currently unclear whether the elevated DSBs observed in *LigIV*^{Y2988C} mice arise directly from replication-associated DSBs or, for example, arise as a consequence of the high metabolic rate in these cells.

As mentioned above NBS and RAD50-deficient patients present with a similar microcephalic phenotype to LIG4 Syndrome patients [82]. However, NBS patient cells, like Artemis defective cells, have a mild DSB repair defect. Although the precise role is still controversial, there is evidence that the MRN complex is also involved in promoting recovery from replication stalling. Details of the basis underlying the microcephaly phenotype in NBS or RAD50-deficiency is, therefore, likely to be somewhat distinct to LIG4 Syndrome, although rapid replication and sensitive apoptotic activation most likely causally contribute to microcephaly in all the disorders.

The markedly impaired pre and post-natal neuronal development observed in one DNA-PKcs patient likely also arises as a consequence of high apoptosis caused by persistent, unrepaired DSBs during neurogenesis. It is possible also that uncapped telomeres, which could arise if telomere length is not efficiently maintained, could additionally signal to the apoptotic machinery. The post natal impact is unlikely to be a consequence of sensitively activated apoptosis but this has not been thoroughly examined.

Finally, a diminished ability to generate neurons due to cell loss leads to a diminished size of the cerebellum. This renders the appearance of a pronounced nose and underlies the dysmorphic facial features also observed in LIG4 Syndrome and XLF-deficient patients, a feature also observed in Seckel Syndrome [98]. The growth delay observed in NHEJ-deficient patients could be the result of a similar impact of unrepaired DSBs on cell loss (via apoptosis or merely a failure to replicate) in other stem cell compartments.

7. How does our knowledge enhance patient care

RS-SCID patients can be identified by assessment of their cellular radiosensitivity or their capacity to repair DSBs. Increasingly, sequencing of candidate genes is employed to identify patients. The provision of a diagnosis is normally psychologically beneficial for the patient or their family. However, there are additional benefits to providing a diagnosis.

Currently, SCID can be readily treated by bone marrow transplantation (BMT), which although costly has a high success rate. However, previous studies have shown that RS-SCID patients and particularly LIG4 Syndrome patients can over-react to the conditioning regime employed to avoid a host-graft response [99]. Alternative regimes which avoid the use of DSB inducing agents have been developed for such patients and found to be highly efficient [100]. The long term outcome for Artemis deficient patients to BMT appears less positive than for other SCID conditions, and such knowledge can enhance the treatment pre and post BMT [65,101] Thus, providing an RS-SCID diagnosis can allow the optimisation of conditioning regimes as well as post transplantation care. Further, there appears to be an elevated incidence of lymphoid leukaemias in some of the RS-SCID patients. Thus, knowledge of a patient's genetic defect can be beneficial in deciding if BMT is the preferred treatment and/or if radiotherapy can be exploited during treatment. Indeed, the first LIG4 Syndrome patient identified showed a marked over-response to radiotherapy [44]. Although BMT can relieve the immunodeficiency of RS-SCID, all the non-immune somatic cells will retain DSB repair defect and potentially enhanced genomic instability. Such patients may need close surveillance for the onset of cancer. Although there is currently no evidence that such patients respond abnormally to the low dose of radiation employed during diagnostic X-ray exposures and computed tomography (CT) scanning, there remains a strong possibility that this could arise, with patients possibly enduring an enhanced likelihood of radiation-induced carcinogenesis. The benefits versus harmful effects of such exposures need to be carefully weighed when caring for such patients. Finally, although the diagnosis of LIG4 Syndrome, XLF-defects and null-Artemis patients is efficient, leaky Artemis deficiency is harder to identify; cellular radiosensitivity without defective DSB repair or with cell cycle stage specific DSB repair defects have been reported [67,72].

Given the benefits of diagnosis, there is scope for enhancing the diagnostic procedures. One approach is the screening of newborn SCID patients using the T cell receptor excision circle (TREC) assay, which detects an intermediate formed during V(D)J recombination [102]. Another procedure has the potential to enhance diagnosis of patients with mild or cell cycle-specific DSB repair defects by enhancing the efficacy of the current approach of analysis, which involves the enumeration of γH2AX foci [103]. In summary, the diagnosis of RS-SCID patients has the potential to enhance their care and treatment, and diagnostic procedures, particularly those allowing the rapid identification of hypomorphic Artemis CID patients, need to be optimised.

8. Concluding remarks

Although the double helical structure of DNA suggested a stable structure endowed with the capacity to maintain a cell's genetic information and transfer it to daughter cells, we now appreciate that the DNA molecule incurs a substantial level of damage. Thus, repair mechanisms are critical for maintaining the DNA stability. The development of the immune response necessitates the generation of genetic diversity and exploits the DNA damage response proteins. A substantial number of patients impaired in their ability to repair DSBs and consequently diminished in their capacity to develop an immune response has now been identified. The features of such patients have additionally revealed a significant role for the major DSB rejoining process, NHEJ, during neurogenesis. The patients identified to date likely represent the extreme spectrum of the impact of loss or deficiency of NHEJ proteins on human health. It is likely that milder defects confer somewhat less dramatic but distinct phenotypes, which could be substantially more common. This is exemplified by "leaky" Artemis deficient patients, where

progressive immunodeficiency has been reported (Table 3). Now that we have significant insight into this extreme end of the spectrum, further work is required to identify the clinical impact of less impacting mutational changes. Our insight has already enhanced the care of RS-SCID patients but further work is required to optimise their diagnosis as well as their long term outcome.

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